A METHOD OF DETERMINING SENSITIVITY OF RICKETTSIA BURNETI TO ANTIBIOTICS IN VITRO

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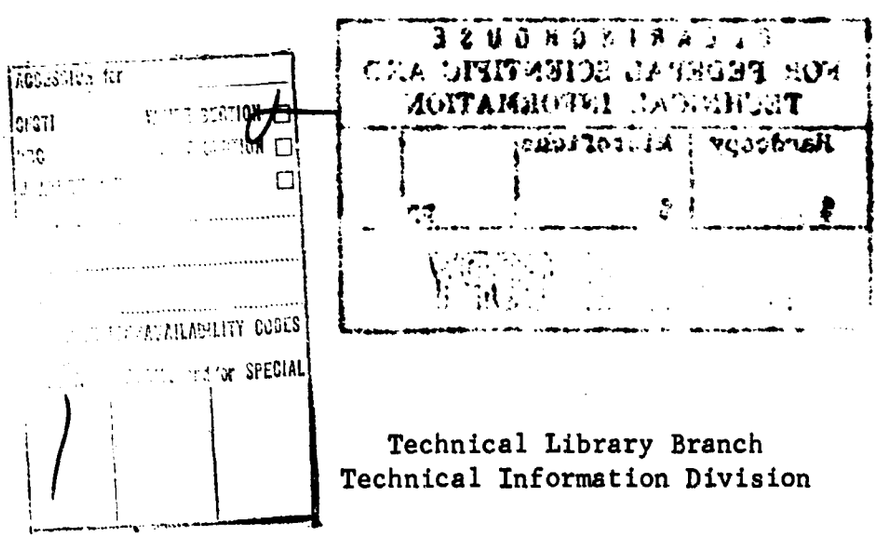

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A METHOD OF DETERMINING SENSITIVITY OF RICKETTSIA BURNETI TO ANTIBIOTICS IN VITRO

/Following is the translation of an article by Ye. P. Savitskaya in the Russian-language journal Antibiotiki (Antiobiotics), Vol 8, No 6, 1963, pages 512-516./

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Several simple methods are available in determining the sensitivity of bacteria to antibiotics in vitro: paper discs, series solutions, phase-contrast microscopy, but not one of them is suitable for a large group of cytotropic microorganisms known under the general name of Rickettsia. Therefore, experiments to determine Rickettsia sensitivity to antibacterial preparations have been carried out either on animals or in chick embryos (1-7).

At present, the effect of antibiotics and other inhibitors on various species of Rickettsia have begun to be studied in vitro in tissue cultures (8).

A method has been proposed of studying the effect of antibiotics on the psitacossis and Rickettsia Burneti viruses in vitro in tissue culture using cinemicrophotography to record the action of oxytetracycline (9). This is a very graphic method, however, it requires fairly complex equipmentation of the laboratory and therefore has not found broad use.

The aim of this study is to develop a simple and more accessible method of determining in vitro the sensitivity of Rickettsia Burneti to antibiotics in order to select the most effective antibiotic.

Methods

Rickettsia Burneti is cultivated in a monolayer culture of fibroblasts obtained by tryptic digestion of 9-10-day old chick embryo. The medium for the cultivation was of the following formulation:

1) synthetic medium 199 -- 30 %; 2) Tirode salt solutions -- 50 %; 3) inactivated calf serum -- 10 %; 4) cellular suspensions -- 10 %; 5) penicillin -- 100 units/ml. One ml of medium contained 155,000-160,000 cells.

The cultures were prepared in ordinary test tubes 180 X 18 mm in size containing 1.6 ml of medium and on cover slides 13 X 24 mm in size, placed on the flat side in small test gubes (10) containing 1.2 ml of medium.

Under these conditions of cultivation (at 36°), the fibroblasts formed a continuous layer by the third-fourth day. At this time, a partially purified suspension of Rickettsia Burneti (Grit strain) was added to the tubes. The mixture was cultivated in chick embryo yolk sacs. The eggs were opened by the fifth-sixth day after infection, as the chick embryos succumbed, the yolk sacs were removed with maximum content of Rickettsia, checked for sterility, and frozen at -25°.

On the day of the experiment, the frozen sacs were rapidly thawed in warm water (35°) and a 10 % suspension in saccharose-glutamate medium (SG) /See Note/

(/NOTE/ The SG medium is of the following formulation: glutamic acid -- 0.005 M, saccharose -- 0.218 M, KH_2PO_4 -- 0.0038 M, K_2HPO_4 -- 0.0072 M, pH 7.0-7.2 (brought to this pH with 1 N NaOH, sterilized in flowing steam at 100° for 40 minutes or at 120° for 30 minutes).

The tissue suspension was partially purified by centrifuging twice for 10 minutes at 3000 rpm.

For Rickettsia infection of the fibroblast cultures, the medium was removed from the test tubes and replaced with fresh (formulation as before) medium containing 10 % Rickettsia suspension. In this way, 1 ml of the medium contained 1/100 of the Rickettsia-infected yolk sac. For the controls, part of the cultures remained uninfected with Rickettsia, and in their place an equal amount of suspension of normal yolk sac in the SG medium was added. The cultures containing Rickettsia were placed in a thermostat at 35°. Multiplication of Rickettsia Burneti began from the second-third day and was accompanied by morphological changes of the fibroblasts -- from elongated polygonal cells they became circular. Some of the cells gradually enlarged. The extent of Rickettsia propagation in the culture was determined by the method of counting 100 cells and recording the Rickettsia accumulated in each cell (11). For this purpose, 1.6 ml of an 0.25 % versene solution was added to the culture in a large test tube, shaken vigorously, and the test tube left stand inclined for 15-20 minutes in a thermostat. Under the effect of Versene and the mechanical action, the cellular layer was shaken off the glass surface and broken up into individual cells. Four thin smears were made from this suspension (two per single slide). The smears were left in a thermostat until dried completely, then they were fixed in the flame of a burner and stained with carbolic fuchsine, decolorized with citric acid, and stained with methylene blue following the Zdrodovskiy method (12). The Rickettsia stained red was easily visible against a background of blue cells.

To determine the extent to which cells have been infected with Rickettsia, 100 well stained whole cells were examined (50 cells in a single smear and 50 in another) and evaluated in the following scheme: 0 -- no rickettsia in the cell; + -- from 1 to 5 Rickettsia in the cell; ++ -- from 6-20; +++ -- more than 20 Rickettsia in the cell.

For high reliability of the results, the affected cells were counted by two researchers in two different preparations; the results of the calculation were totaled and the average value derived. For example, multiplication of Rickettsia Burneti in fibroblasts (in percentages) by days appears as follows:

a) На 3-й день	b) На 6-й день	c) На 9-й день	d) На 11-й день	e) На 18-й день
0 — 41	0—42	0—39	0—14	0—6
+ — 42,5	+—20	+— 14,5	+—24	+—8
++ — 8	++—6	++—10	++—13	++—6
+++ — 8,5	+++—32	+++—36,5	+++—49	+++—80

LEGEND: a) on the third day; b) on the sixth day; c) on the ninth day; d) on the eleventh day; e) on the eighteenth day.

On the third day following Rickettsia infection of the culture the medium was changed and placed with fresh solution containing the antibiotic being studied. For controls, an equal amount of culture was left without antibiotic.

On the second-third day following addition of the antibiotics, Bersene was used to smear the cells from one test tube and from one control group in order to estimate the number of cells containing Rickettsia by +++. The difference in the number of cells infected at +++ by Rickettsia in the control and in the experimental culture were an indication of the antiricketal effect of the antibiotic.

On the fifth day following addition of antibiotics, the medium in the cultures was replaced by fresh, containing the same amount of preparation. In order to trace the effect of the antibiotic, the cells were counted several times after two-three days.

During the same time, when the cells were being counted, preparations were prepared in toto for microscopic examination of the cultures on covered glasses. The latter were withdrawn from test tubes, rinsed in a warm Tirode solution in order to wash the nutrient medium, fixed and stained by one of the methods suitable for staininn Rickettsia (according to Romanovskiy, Zdrodovskiy, Gimz, etc.).

Experimental Results

In the first experiment, chlortetracycline was added to the Rickettsia Burneti culture, which antibiotic as is known (6) exhibits Rickettsia-static properties to this species of Rickettsia. The dose of

Figure 1. Culture of chick embryo fibroblasts infected with Rickettsia Burneti, after action of an effective antibiotic. Stain according to Romanovskiy. Magnification: 1000 X.

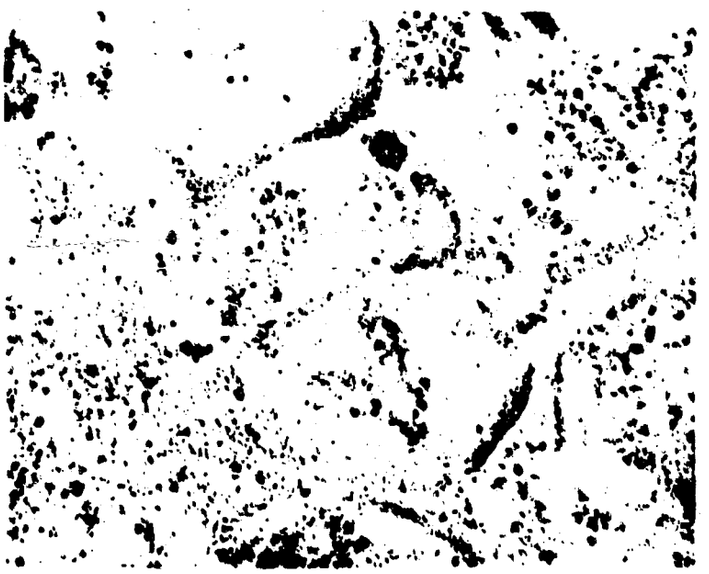

Figure 2. Chick embryo fibroblast culture infected with Rickettsia Burneti, following the action of an inactive antibiotic. Staining according to Romanovskiy. Magnification: 1000 X.

chlortetracycline in 1 ml of medium was 20 micrograms. During the course of the experiment, the medium containing the antibiotic was replaced with fresh solution once more on the fifth day following the first addition of preparation; the Rickettsia content in the cells was counted three times, on the third, fifth, and seventh day following initial addition of the antibiotic. As a result, the following data was obtained on the number of cells infected by +++ Rickettsia (in percentages of the control and experimental cultures).

	На 3-й день (a)	На 5-й день (b)	На 7-й день (c)
Контроль (d)	64	54,5	40,5
Опыт (e)	20	14	8

LEGEND: a) on the third day; b) on the fifth day; c) on the seventh day; d) control; e) experiment.

TABLE 1

Effect of chlortetracycline on multiplication of R. burneti in vitro in cell cultures

(a) Риккетсии Бернета	(b) Доза антибиотика (в γ/мл)	(c) Процент клеток, зараженных риккетсиями на +++
(d) Контрольная	—	52,5
(e) С хлортетрациклином	20	9,5
Контрольная	—	40,5
С хлортетрациклином	25	3
Контрольная	—	80
С хлортетрациклином	25	4
Контрольная	—	10,5[1]
С хлортетрациклином	25	0
Контрольная	—	84
С хлортетрациклином	30	1,5

[1] In this experiment the multiplication of Rickettsia Burneti in the control culture was very slight.

LEGEND: a) Rickettsia Burneti; b) dose of antibiotic (in micrograms/ml; c) Percentage of cells infected with Rickettsia to +++; d) Control; e) With chlortetracycline.

To verify the reliability of the results and to determine the sensitivity of the method subsequent experiments were performed with chlortetracycline used in different doses. These results are shown in Table 1.

TABLE 2

Comparative effect of different antibiotics on Rickettsia Burneti

(a) Первая группа				(b) Вторая группа				(c) Третья группа			
(d) Антибиотик	(e) Доза (в γ/мл)	(f) Число клеток, зараженных риккетсиями на +++ (в %)		(d) Антибиотик	(e) Доза (в γ/мл)	(f) Число клеток, зараженных риккетсиями на +++ (в %)		(d) Антибиотик	(e) Доза (в γ/мл)	(f) Число клеток, зараженных риккетсиями на +++ (в %)	
		(g) Контроль	(h) Опыт			(g) Контроль	(h) Опыт			(g) Контроль	(h) Опыт
Эритромицин (i)	30	54	1	Неомицин (v)	30	54	15	Канамицин (w)	30	30,5	8,5
Хлортетрациклин (j)	30	54	1,5	Мономицин (r)	30	54	18	Гризеин (u)	30	30,5	10
Тетрациклин + нистатин (k)	30	54	4	Олеандомицин (s)	30	54	23,5	Спонтин (ристоцетин) (v)	30	30,5	13,5
Тетрациклин (l)	30	54	8					Канамицин (w)	30	30	12
Окситетрациклин (m)	30	54	8					Мицерин (x)	30	30	63
Сецазин (n)	30	54	5,5								
Левомицетин (o)	30	54	8								
Синтомицин (p)	30	54	8								

[1] In this experiment very slight multiplication of Rickettsia was observed in the control culture.

LEGEND: a) first group; b) second group; c) third group; d) antibiotic; e) small dose (in micrograms/ml); f) number of cells infected with Rickettsia to +++ (in percent); g) control; h) experiment; i) Erythromycin; j) Chlortetracycline; k) Tetracycline + nystatine; l) Tetracycline; m) Oxytetracycline; n) Secazine; o) Levomycetin; p) Sintomycine; q) Novobiocyn; r) Monomycin; s) Oleandomycin; t) Colimycin; u) Grisemin; v) Spontin (ristocetin); w) Canamycin; x) Micerin.

As we can see from Table 1 the method of estimating cells infected with +++ Rickettsia in the control culture and in the test culture can be used to detect fluctuations in the effect of antibiotics of different doses. Thus, in experiment no. 1 at a 20 microgram/ml dose of tetracycline the percentage of infected cells to +++ was 9.5, while in experiment 5 the antibiotic dose of 30 microgram/ml reduced the percentage of infected cells to 1.5 (together with equalized controls), which pointed to the sufficient sensitivity of this proposed method.

Upon comparing the activity of chlortetracycline and sintomycin the latter proved less active: on the 18th day after addition of antibiotics in the control 80 per cent of the cells infected with Rickettsia to +++ amounted to 9.5 per cent in the culture containing sintomycin, and 4 per cent in the culture containing chlortetracycline.

Microscopic investigations of preparations in total revealed the presence of cytopathogenic effect of Rickettsia Burneti on the fibroblast culture, which was manifest in change of the usual form of cells; they were surrounded, the nucleus was shifted toward the side, and the cell resembled a sphere surrounded by a thick envelope. Changes in the cells were noted on the third-fourth day after infection with the Rickettsia culture, that is, before the onset of their copious multiplication.

Under the effect of chlortetracycline not only was the amount of Rickettsia in the cell cytoplasm observed to decrease, but the cells themselves again acquired the form usual for fibroblasts, and a "recovery" of the cells seem to take place with the restoration of the usual morphology (Figures 1 and 2). This is so characteristic a picture that from a few morphological changes the favorable effect of the antibiotics can be estimated.

Based on these experiments we concluded that the suggested method can be used in studying the effect of antibiotics on Rickettsia Burneti cultures. In order to indicate the suitability of this method for selecting antibiotics active with respect to Rickettsia Burneti, 16 coded preparations were studied, obtained by number from another laboratory /See Note/. During the experiment, after the addition of antiotics the medium was replaced once more on the fifth day, the effective cells were counted always during the same period: on the third, sixth, and ninth day. All of the antibiotic was added to the medium in doses of 30-50 micrograms/ml.

(/NOTE/ I am thankful for A. F. Moros for obtaining the antibiotic for this study.)

After determination of the effect of unknown preparations we obtained their coding, and on the basis of the data obtained have prepared the composite Table 2 based on certain experiments of which it is known that the method described can select and determine the antibiotics most active with respect to Rickettsia Burneti.

In the first group are antibiotics exhibiting a Rickettostatic action. As can be seen from Table 1, erythromycin evidences the most active response to the Rickettsia Burneti.

Monomycin, novobiocyn, and oleandomycin, exhibiting weak Rickettostatic effect belong to the second group; the third group includes colimycin, grisemin, spontin, canamycin, and micerin, which have been inactive with respect to Rickettsia Burneti.

Conclusions

1. A method has been suggested for determining the activity of antibiotics with respect to Rickettsia Burneti in vitro in cells of chicken fibroblasts. The activity of the antibiotics is determined from the difference in the amount of cells intently affected by Rickettsia in the control culture and in the culture undergoing the action of antibiotics.

2. The effect of antibiotics is manifest in a sizable reduction of the propagation of Rickettsia Burneti in the cytoplasm of fibroblasts, a reduction in number of Rickettsia-affected cells, and in the elimination of the cytopathogenic effect of Rickettsia on cells of tissue cultures.

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